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Comparison of site-specific coupling chemistry for antibody immobilization on different solid supports

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ABSTRACT

Silica is an important chromatographic support for high-performance affinity chromatography due to its high mechanical stability. However, silica is very different from traditional gel chromatographic materials, such as agarose, dextran, and polyacrylamide, with respect to many chemical and physical properties. Thus, it is expected that different immobilization techniques must be used for orienting the immobilized ligand on the surfaces of the two substrates. In this study, a site-specific coupling chemistry for immobilization of antibodies on modified silica surfaces and on agarose gels was investigated. The effects of substrates on the orientations of immobilized native and partially denatured antibodies was deduced. An important conclusion is that non-covalent interactions (physical adsorption) dominate the orientation of immobilized antibodies on silica surfaces.

INTRODUCTION

Due to their high specificity, antibodies immobilized on chromatographic supports have been widely used for purification of molecules since the early 1970s [1]. Numerous coupling chemistries for the immobilization of antibodies have been developed and reviewed [2,3]. Most coupling chemistries rely on the random coupling of antibodies through the amino groups of lysine residues. This often results in a decrease in antigen binding capacity (AgBC) due to improper orientation of the

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antibody molecules on the solid surfaces. To overcome these problems, two approaches for site-specific immobilization of antibodies have been reported recently. In the first approach, antibodies are immobilized to hydrazide-activated supports via their oxidized carbohydrates by forming covalent hydrazone bonds. Immunoglobulins contain carbohydrate moieties linked to the CH_2 domain of the Fc fragments. Under mild conditions, the hydroxyl groups of the carbohydrates can be oxidized to aldehyde groups by sodium periodate without significantly impairing the active sites of the antibody [4–6]. Another method is to immobilize Fab′ fragments via thiol groups at the hinge region [6–8]. This can be done by first making $F(ab)'_2$ with pepsin digestion, followed by reduction of the disulfide bond which links the two Fab′ fragments by dithiothreitol. The free thiol groups can then be coupled to maleimide-activated supports.

When antibodies are immobilized to agarose gels, both site-specific coupling methods result in an increase in AgBC over non-selective coupling methods. However, agarose gel is not an ideal support for high-performance affinity chromatography (HPAC) because of its low mechanical stability. Recently, it has been suggested that silica is a promising material for HPAC due to its inherent mechanical stability, which provides good flow characteristics even under high pressure [9].

However, silica and agarose exhibit completely different chemical and physical properties. For example, agarose gel is a soft organic material with high water content, while silica is a hard inorganic material. From the point of view of antibody immobilization, there is one important difference between the two materials which has been ignored so far and needs to be examined, protein adsorption or resistance properties. Why are these properties important for site-specific couping of antibodies? Examine Fig. 1.

In the upper part of Fig. 1, materials used for chromatographic supports are roughly classified into three categories, according to their protein resistance properties. The first category includes natural and synthetic uncharged hydrogels, such as agarose, dextran, and polyacrylamide, which exhibit low protein adsorption properties. Although a number of explanations have been offered as to why proteins exhibit weak interactions with these materials[10], the exact mechanism is still unclear. The second category includes materials such as silica, polystyrene, and polyethylene, which exhibit strong protein adsorption. Depending on the nature of the surface, protein, and solution medium, the protein is adsorbed via ionic interactions, hydrophobic effects, polar–polar interactions, Van der Waals forces, or, most probably, a combination of all such interactions [10,11]. An enormous amount of work has been reported in the literature on the protein adsorption to various substrates [11,12]. The third category consists of the materials which have the protein adsorption properties somewhat between the two types of materials discussed above.

Site-specific coupling of a protein to agarose (left) and silica (right) surfaces is schematically shown in the lower part of Fig. 1. Examine the agarose situation first: the open circle on the hydrogel surface represents a functional group which can form a covalent bond with a specific site on the protein (shown by the closed circle on the protein). Our hypothetical protein is a single polypeptide chain and consists of two domains, whose surface includes regions of hydrophobic, charged, or polar character. According to the collision theory of bimolecular reactions, the protein molecule must first approach and collide with the surface before the covalent bond can be formed.

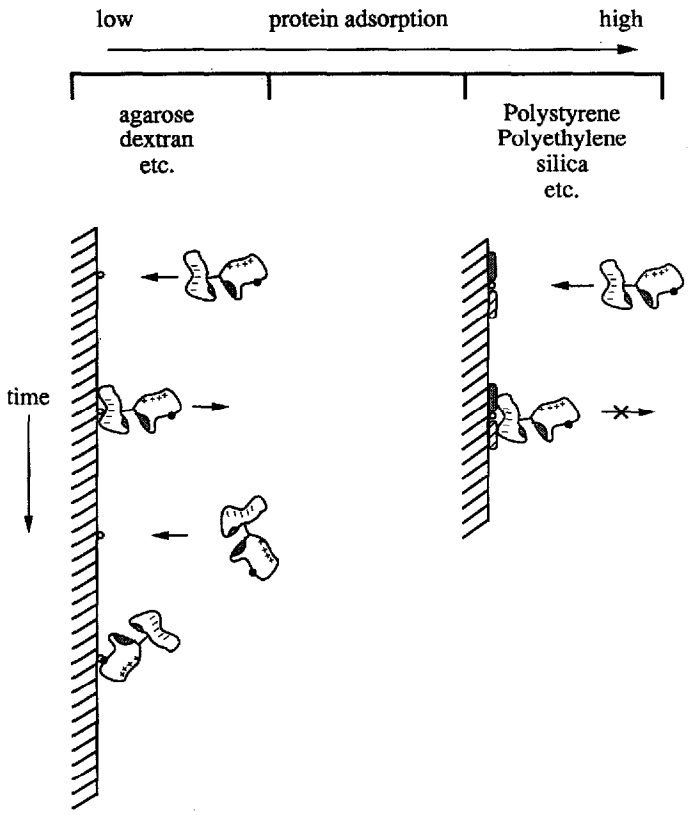

Fig. 1. Schematic representation for the concept of antibody immobilization on different types of solid supports.

The first collision may not be of proper orientation for the formation of a covalent bond. Since agarose has very weak non-covalent interactions with the protein, the protein will then diffuse away from the surface and other proteins will collide with the surface in different orientations. After a number of collisions, protein finally approaches the surface with an orientation suitable for formation of chemical bonds. This surface coupling process is similar to solution reactions except that one of the reactants is a stationary phase.

In the case of silica (right, Fig. 1), non-covalent adsorption sites (represented by the filled squares) are presented on the silica surface, and probably influence the site-specific coupling of the protein. Therefore, in order to understand site-specific coupling to silica surfaces, protein adsorption must be examined. The general concepts of protein adsorption currently accepted have recently been reviewed by Andrade and co-workers [10–12]. In summary, for a one-component system, a protein of given bulk concentration diffuses to and collides with the interface. If the interaction forces and contact area are large enough, the protein stays on the surface for a certain residence time, probably in the range of milliseconds to seconds, while on the surface, the protein may begin surface "denaturation". With increasing contact time, the probability for

desorption decreases. Eventually, the protein may become irreversibly adsorbed on the surface in a completely or partially denatured conformation. Therefore, one could expect that on the silica surfaces the formation of chemical bonds is secondary to physical adsorption.

From the above discussion, it seems that, depending on the types of substrates, different immobilization strategies should be employed for controlling the orientation of the immobilized protein. But, how can we experimentally prove it? We have previously shown that the immobilization of the partially denatured antibodies on silanized silica surfaces resulted in higher AgBC and higher antibody surface concentrations than for the native antibody [13]. The antibody immobilization methods used in the previous study were physical adsorption and non-site-specific chemical coupling. In this study we have immobilized antibodies on different supports to investigate the substrate effect on the site-specific coupling for antibody immobilization. Hydrazide-derivatized agarose and silica were used to immobilize oxidized and non-oxidized native and partially denatured antibodies.

EXPERIMENTAL

Cleaning of silica samples

All silica samples were cut from 2.5 cm × 2.5 cm × 0.1 cm fused-silica slides (CO grade, ESCO) and the edges were finely polished. The size of the sample was 1.1 cm × 0.95 cm × 0.1 cm and fits into a 13 × 75 mm culture tube (Fisher) in which all surface reactions took place at room temperature. Cleaning of the silica samples has been described elsewhere [13].

Silanization of cleaned silica samples

Two silane reagents were used in this study: γ-glycidoxypropyltrimethoxysilane (GOPS, Aldrich) and dimethyldichlorosilane (DDS, Petrarch). The silica chips were reacted with a dry toluene solution of GOPS (2% GOPS, 0.2% triethylamine and 97.8% dry toluene, v/v) [14] or dry toluene solution of DDS (10% DDS and 90% dry toluene, v/v) for 1 h and 30 min at room temperature, respectively. The GOPS samples were rinsed with dry acetone and the DDS samples were rinsed with absolute ethanol. The chips were then cured in a vacuum oven (which has been flushed with nitrogen three times) at 120–130°C for 1 h. The epoxide groups of GOPS on the silica surfaces were then reacted with a 10% hydrazine solution (E. Merck) in methanol for 3.5 h at room temperature, followed by rinsing with deionized water first and then absolute ethanol and dried in a vacuum oven for 1 h at 120–130°C. The hydrazide-treated silica (Si-Hz) surfaces were analyzed by electron spectroscopy for chemical analysis (ESCA) to confirm the occurrence of the surface modification.

Antibody and antigen system

The polyclonal goat anti-human serum albumin (anti-HSA) immunoglobulin G (IgG) fraction was purchased from Cappel Labs. Crystallized human serum albumin (HSA) was purchsed from Miles Diagnostics.

Radiolabelling of proteins

The anti-HSA or HSA was labelled with carrier-free ^{125}I (100 mCi/ml,

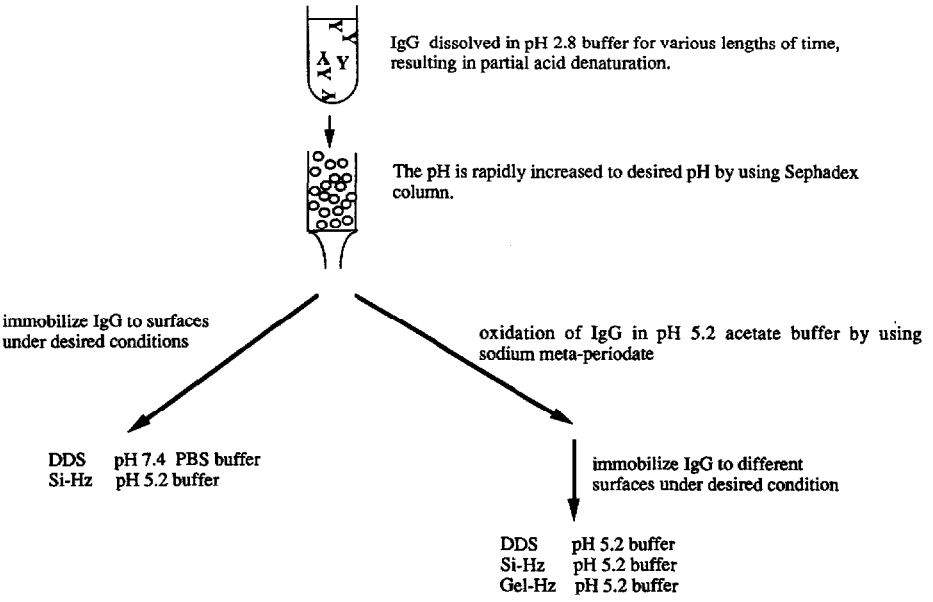

Fig. 2. Schematic representation of the antibody denaturation and immobilization procedures. DDS = dimethyldichlorosilane-treated silica surface; Si-HZ = hydrazide-treated silica surface; Gel-Hz = hydrazide-treated agarose gel.

Amersham) by the chloramine-T method as described by Lin *et al.* [13]. The final concentration of [^{125}I]protein solution was measured in a UV–visible spectrophotometer (Beckman, Model 35) at 280 nm. Values of 0.54 and 66,000 were used for the absorption extinction coefficient ($E^{0.1\%}$) and molecular weight (MW) of HSA, respectively, while for IgG the values of $E^{0.1\%}$ and MW used were 1.35 and 150,000, respectively. Labelling efficiency was determined by precipitating proteins with 20% trichloroacetic acid (TCA, Sigma) in the presence of bovine serum albumin (BSA) as carrier protein. The amount of iodide bound to protein was determined by subtracting the counts in the supernatant from the total counts in solution.

Preparation of the partially denatured antibodies

Antibody was partially denatured by dissolving approximately 5 mg of anti-HSA in 0.5 ml of 0.1 *M* citric acid–phosphate buffer (pH 2.8) for various lengths of time: 1, 20, 60 and 300 min (Fig. 2). The pH of the antibody solution was then increased by using a PD-10 column (Pharmacia) which has been equilibrated with the buffer used for antibody immobilization.

Oxidation antibodies

The native or partially denatured antibodies were oxidized by sodium meta-periodate (J. T. Baker) in 0.1 *M* acetate buffer pH 5.2 (Fig. 2). Sodium meta-periodate stock solution (30 mg/ml) was added to antibody solution (3 mg/ml) at one tenth the final volume. The mixture was gently mixed for various lengths of time (20, 40 and 60 min) at room temperature and the sodium meta-periodate was then removed using

a desalting column which had been washed and equilibrated with diluted coupling buffer, pH 5.2 acetate buffer.

Immobilization antibodies

Silica. Oxidized or non-oxidized samples of native or partially denatured anti-HSA antibodies were immobilized via either physical adsorption or site-specific chemical coupling. For physical adsorption, three experiments were performed: (1) non-oxidized antibodies in 0.15 *M* phosphate-buffered saline (PBS, pH 7.4) adsorbed onto DDS silica surfaces; (2) non-oxidized antibodies in 0.1 *M* acetate buffer (pH 5.2) adsorbed onto S-Hz surfaces; and (3) the oxidized antibodies in 0.1 *M* acetate buffer (pH 5.2) adsorbed onto DDS silica surfaces. For site-specific coupling, the oxidized antibodies were reacted with the Si-Hz surfaces in 0.1 *M* acetate buffer (pH 5.2.). The adsorption or reaction time for all experiments was 3 h at room temperature followed by rinsing with buffer. The concentration of antibody solutions ranged from 0.7 to 0.9 mg/ml. Surface concentrations of the immobilized antibodies were determined by radiolabelling technique.

Agarose gel. The Affi-Gel hydrazide agarose gel (gel-Hz) purchased from Bio-Rad was used to immobilize anti-HSA antibodies. The gel contained hydrazide groups, and coupling procedures have been described elsewhere [15,16]. Solution concentrations of antibodies ranged from 0.8 to 1.1 mg/ml. The amount of antibody immobilized on the gel beads was determined by measuring the depletion of antibody in bulk solution with a UV–VIS spectrophotometer.

Antibody–antigen binding experiments

The anti-HSA-coated silica and hydrogel samples were incubated with an excess amount of iodinated HSA in PBS for 1 and 2 h at room temperature, respectively. The silica samples were held by forceps and rinsed with PBS gently to remove the weakly adsorbed antigen solution layer. The hydrogel samples were rinsed with PBS more than five times until no iodinated HSA was detected in the rinsing solution. In all antigen binding experiments, an excess amount of BSA was added to the HSA solution to minimize non-specific adsorption. The amount of BSA added was 70–100 times higher than that of HSA.

Fluorescence measurement

Conformation changes of the antibodies resulted from acid treatment or oxidation were characterized by 4,4′-bis[8-(phenylamino)naphthalene-1-sulfonate] (bis-ANS, Molecular Probes). Bis-ANS fluoresces strongly with an emission peak at 515 nm when bound to hydrophobic regions of proteins, whereas the unbound dye is virtually non-fluorescent in aqueous buffer [17,18]. For non-oxidized antibodies, the final concentrations of the antibodies and bis-ANS in PBS were $3 \cdot 10^{-6}$ and $1 \cdot 10^{-5}$ *M*, respectively. For oxidized antibodies, the fluorescence measurements were performed in pH 5.2 acetate buffer in which cross-linking between antibody molecules was prevented. The final concentrations of the antibodies and bis-ANS were $6 \cdot 10^{-6}$ and $1 \cdot 10^{-5}$ *M*, respectively. The fluorescence experiments were performed in a Greg-200 multifrequency fluorometer (ISS, Champaign, IL, U.S.A.). The sample solutions were excited with plane-polarized light at 400 nm from a broadband 200-W xenon high-pressure lamp. The fluorescence at 515 nm was collected at 90° with respect to the incident light.

RESULTS

The AgBC of oxidized and non-oxidized antibodies immobilized on DDS silica surfaces are shown in Fig. 3 as a function of antibody denaturation time. The zero time point is for the native antibody, immobilized without low-pH treatment. Denaturation of antibody in low-pH buffer for 1 min, 20 min, 1 h and 5 h were performed (Fig. 2). Prior to immobilization, the low-pH buffer was exchanged with pH 7.4 PBS buffer for the non-oxidized antibodies or pH 5.2 acetate buffer for the oxidized antibodies.

For non-oxidized antibody, the AgBC after 20 min exposure to low-pH buffer is approximately two times higher than that of the native antibody (0 min) case (Fig. 3a). This increase in antigen binding is probably due to specific interactions because BSA was used to reduce non-specific binding. Furthermore, the AgBC decreases dramatically with increasing denaturation time. After 5 h of exposure to low-pH buffer, the AgBC is slightly lower than that of the native case. Although this set of results has been published previously, it is listed here again for the purpose of comparison [13]. A similar trend was also observed for antibodies which had been oxidized with sodium meta-periodate (Fig. 3b). The AgBC increased with increasing antibody denaturation

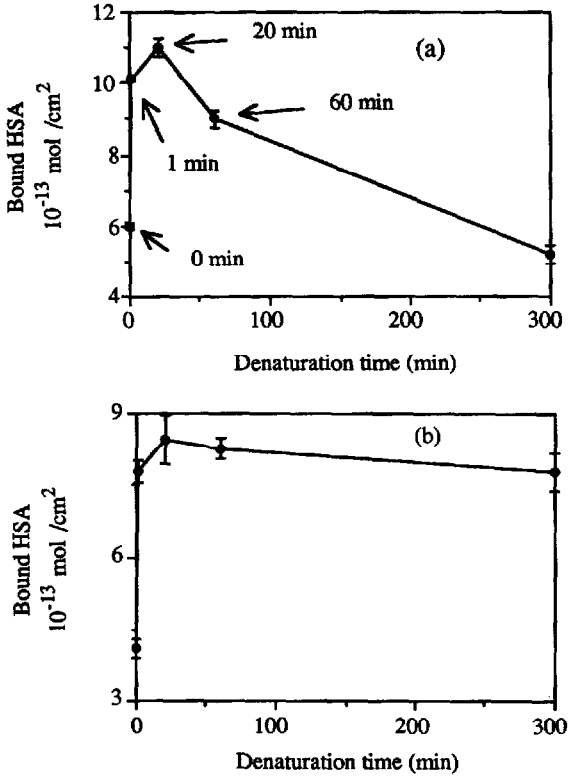

Fig. 3. Antigen binding capacity of immobilized antibody on DDS surfaces as a function of antibody denaturation time: (a) non-oxidized antibody ($n = 3$) (from ref. 13); (b) oxidized antibody ($n > 4$) (n = number of samples). The zero time point is for the native antibody, immobilized without low-pH treatment.

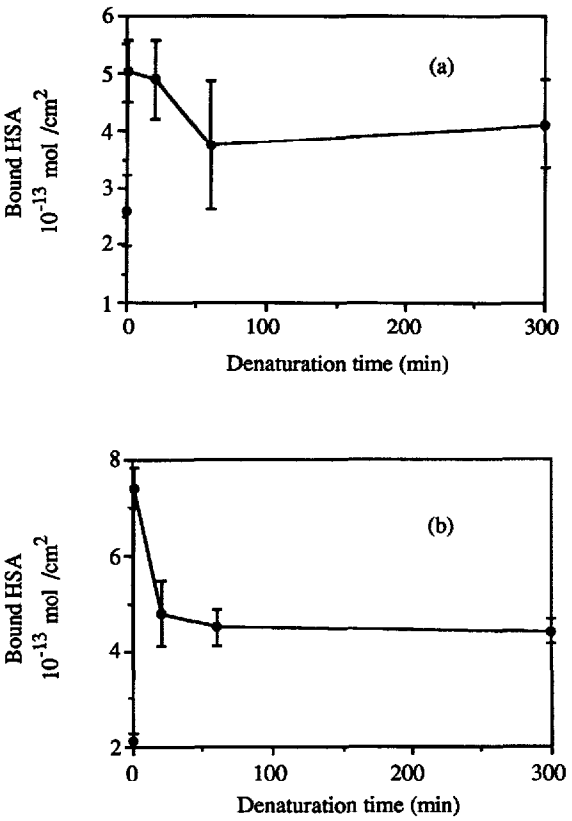

Fig. 4. Antigen binding capacity of immobilized antibody on Si-Hz surfaces as a function of antibody denaturation time: (a) non-oxidized antibody ($n=3$); (b) oxidized antibody ($n=8$).

time and reached a maximum after 20 min. The AgBC was approximately two times higher than that of the native case. At longer times, the AgBC decreased slightly with further denaturation time.

The AgBC of oxidized and non-oxidized antibodies immobilized on Si-Hz surfaces are shown in Fig. 4. The results were similar to those observed on DDS surfaces. The maximum AgBC of both antibodies on Si-HZ surfaces occur after 1 min of low-pH treatment, and they are about two to three times higher than those of the native antibodies. The surface concentrations of non-oxidized and oxidized antibodies immobilized on DDS and Si-Hz surfaces were also determined as a function of antibody denaturation time (Figs. 5 and 6, respectively). In all cases, exposure of antibodies to low-pH solution prior to immobilization dramatically increased antibody surface concentrations.

In contrast to modified silica surfaces, the hydrogel exhibited very good protein resistance [13]. In other words, the antibodies did not adsorb to agarose and can only be immobilized to the beads through covalent bonds. This was demonstrated by incubation of the hydrogel beads with iodinated non-oxidized native and partially

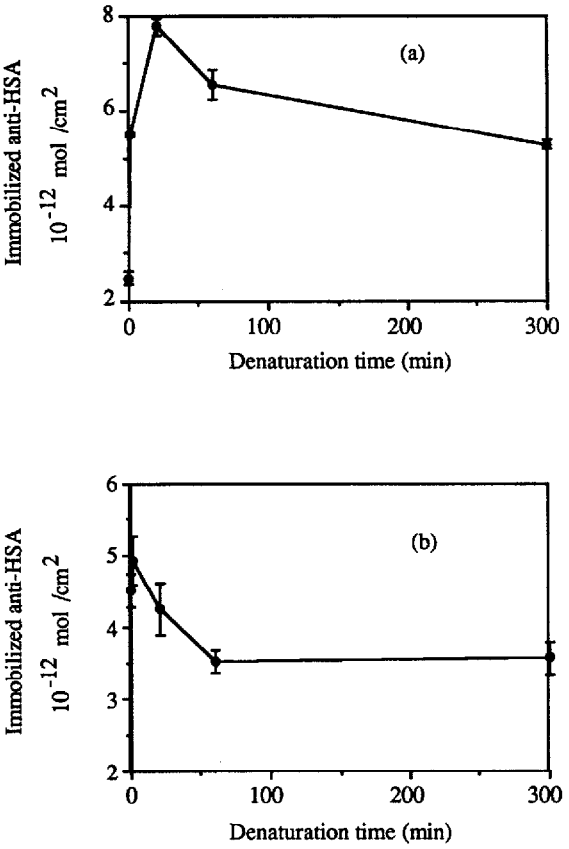

Fig. 5. Surface concentrations of anti-HSA on DDS surfaces as a function of antibody denaturation time: (a) non-oxidized antibody ($n=3$); (b) oxidized antibody ($n>4$).

denatured antibodies (5-h acid treatment). In principle, formation of covalent bonding is not possible in this case. After incubation and extensive rinsing, the beads were counted for antibody surface concentrations. The results show that only a trace of antibody bound to the beads. Therefore, the hydrogel beads were used as control for a surface which exhibits little or no protein adsorption.

The amount of oxidized antibodies and their AgBC immobilized on the Affi-Gel beads were determined as a function of antibody denaturation time (Fig. 7). The results showed that the AgBC and antibody surface concentrations decreased concomitantly with increasing antibody denaturation time. This suggested that the decrease in AgBC is caused by the decrease in antibody surface concentration, rather than the orientation of the immobilized anibody. Furthermore, the initial increase in the AgBC that was observed with short denaturation time on silica samples (Figs. 2–4) was not observed with hydrogel beads, suggesting that different mechanisms were involved in antibody immobilization on the two different types of materials.

It is well known that proteins in low-pH solution undergo conformational

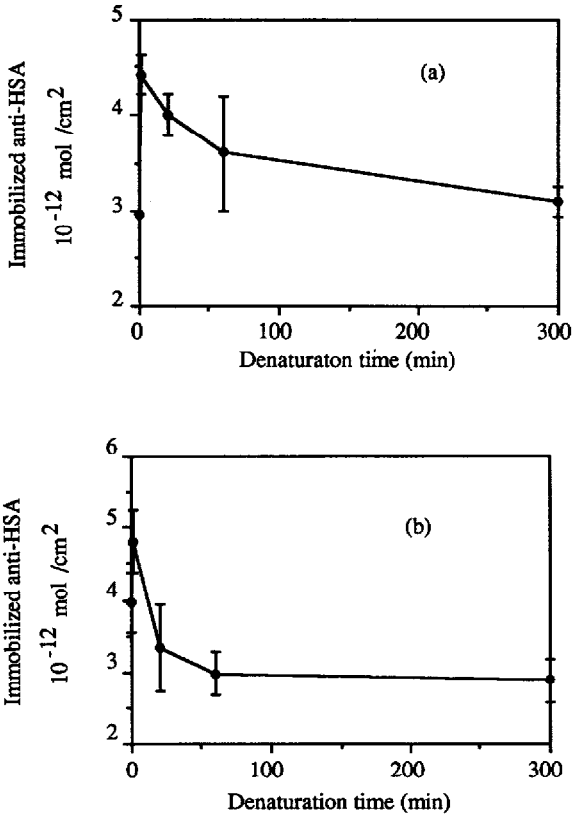

Fig. 6. Surface concentrations of anti-HSA on Si-Hz surfaces as a function of antibody denaturation time: (a) non-oxidized antibody ($n=3$); (b) oxidized antibody ($n=7$).

changes, which in turn affect their adsorption properties. The conformational changes of the partially denatured antibodies were characterized using a fluorescent hydrophobic probe, bis-ANS. The fluorescence spectra of bis-ANS in different antibody solutions are shown in Fig. 8. It can be seen that the fluorescence intensity of bis-ANS increases with increasing antibody denaturation time, indicating conformational changes of the Abs. In Fig. 8, data for the antibody with acid treatment for 5 h are not shown because the fluorescence intensity was too high to measure. The effect of oxidation time on the conformation of antibodies was also characterized by bis-ANS (Fig. 9). The fluorescence spectra for the antibodies oxidized by sodium periodate for 20, 40 and 60 min (curve c and d) overlap each other and are higher than that for the non-oxidized antibody (curve b). This indicates that oxidation processes have changed the conformation of certain parts of the antibody molecule and that the kinetics of the conformational changes are relatively fast (less than 20 min).

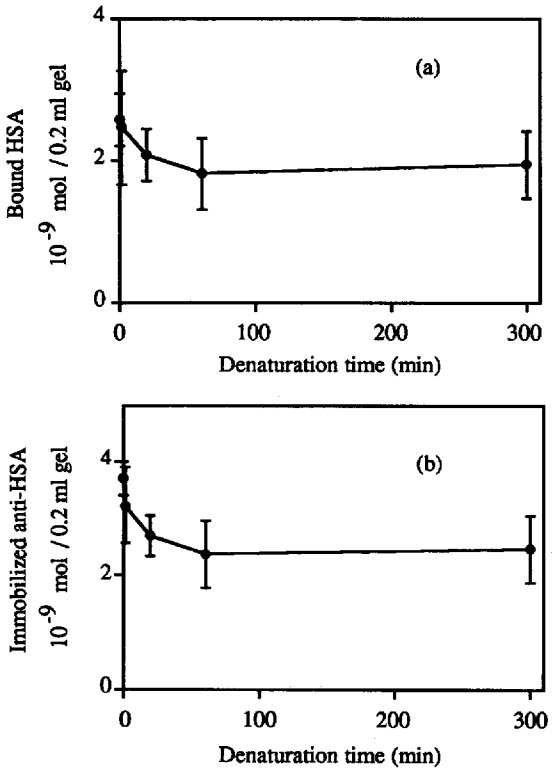

Fig. 7. (a) Antigen binding capacity of immobilized antibody; (b) surface concentrations of anti-HSA on agarose gel as a function of antibody denaturation time; $n=4$ in both experiments.

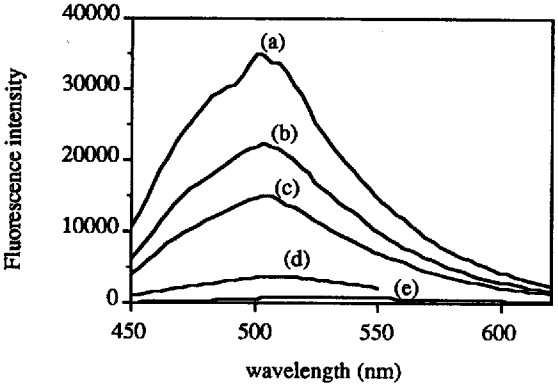

Fig. 8. Fluorescence spectra of bis-ANS binding to the non-oxidized native and partially denatured antibodies in pH 7.4 PBS; bis-ANS in (a) anti-HSA acid treated for 60 min, (b) anti-HSA acid treated for 20 min, (c) anti-HSA acid treated for 1 min, (d) native anti-HSA and (e) PBS.

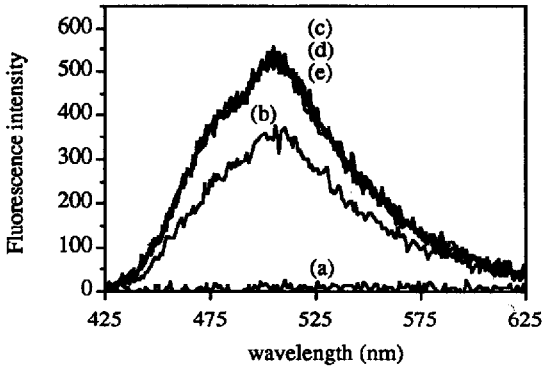

Fig. 9. Fluorescence spectra of bis-ANS binding to anti-HSA as a function of oxidation time in pH 5.2 acetate buffer; bis-ANS in (a) buffer, (b) non-oxidized native anti-HSA and (c–e) anti-HSA oxidized for 20, 40 and 60 min, respectively.

DISCUSSION

Oxidized or non-oxidized native and partially denatured antibodies were immobilized on three different surfaces via physical adsorption or chemical coupling. From the chemistry point of view, the hydrazide on the surfaces can react with aldehyde groups of oxidized carbohydrates in Fc fragments, resulting in site-specific coupling. Our results indicate that this chemistry works very well on the agarose gels. First, since only oxidized antibodies could be immobilized to agarose gels, immobilization was due exclusively to covalent coupling (Fig. 7). Second, the apparent activities of all immobilized antibodies using hydrazide chemistry on the agarose gels were 75 $\pm$ 3%, calculated assuming that only half of the IgG is the specific antibody, according to the specification provided by the company (Fig. 7). This value is consistent with other researchers' finding and is generally two to four times higher than that of non-site-specific coupling methods, such as cyanogen bromide-activated and N-hydroxysuccinimide ester-activated agarose gels [4,5].

Does hydrazide chemistry work as well on silica surfaces as on the agarose gels? From the results shown in Fig. 4b and 6b, the apparent activities of the immobilized oxidized antibodies on Si-Hz surfaces exhibited values ranging from 5 to 15%, which are substantially lower than that on the agarose gels. This is because the orientation of the antibodies on Si-Hz surfaces was controlled by the non-covalent interactions between the surfaces and antibodies rather than by site-specific coupling chemistry. This hypothesis is supported by the following experimental observations. First, in the control experiments for Si-Hz surfaces (Fig. 4a and 6a), the non-oxidized antibodies were strongly adsorbed onto the surfaces and showed approximately the same degree of AgBC as oxidized antibodies. This means that even though chemical bonds were formed between the antibody and the surfaces, the rest of the antibody molecule could still interact with non-specific adsorption sites on the surface and may have undergone surface denaturation. As a consequence, there is no advantage of using site-specific coupling with silica surfaces. Furthermore, since antibodies tend to adsorb irreversibly to silica surfaces, the chance of the adsorbed molecule having the right orientation for

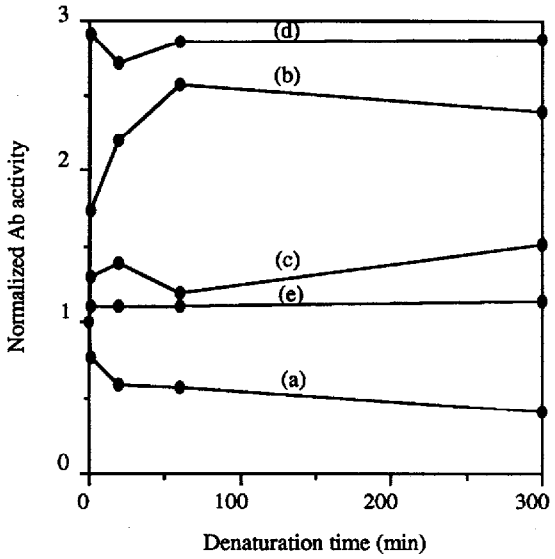

Fig. 10. Normalized antibody (Ab) activity on five different surfaces: (a) non-oxidized anti-HSA immobilized on DDS surfaces; (b) oxidized anti-HSA immobilized on DDS surfaces; (c) non-oxidized anti-HSA immobilized on Si-Hz surfaces; (d) oxidized anti-HSA immobilized on Si-Hz surfaces; (e) oxidized anti-HSA immobilized on Affi-Hz gel.

site-specific coupling is low. Thus, most of the antibodies were immobilized primarily via physical adsorption.

Second, if physical adsorption dominates the orientation of the antibody on silica surfaces, in principle it is possible to manipulate the orientation by altering the adsorption properties of the antibody. This concept is demonstrated by using the partially denatured antibodies. From the fluorescence experiments (Fig. 8), the results show that exposure of hydrophobic regions of the antibody increases with increasing acid denaturation time. As a result, acid treatment prior to immobilization has a significant influence on both antibody surface concentrations and AgBC, as shown in Figs. 2–5. Oxidation can also change the conformation of antibodies (Fig. 9), which in turn affects their adsorption properties, as observed on the non-functionalized DDS surfaces (Figs. 3 and 5). We have previously suggested that the increase in antibody surface concentrations was due to the high affinity of the denatured regions for the surfaces [13]. This phenomenon is analogous to the "Vroman effect" competitive adsorption between proteins [10]. In our case, the competitive adsorption is between the denatured and non-denatured regions of the antibody.

On the agarose gels, antibody surface concentration decreases with increasing denaturation time (Fig. 7). Since non-oxidized antibodies did not adsorb to agarose gels, we believe that the decrease in amount of immobilized antibody is due to the decrease in accessibility of the carbohydrate groups after acid treatment.

Finally, Fig. 10 shows the plot of normalized activity of immobilized antibody as a function of antibody denaturation time for all immobilization experiments. The normalized values were calculated by

$$\text{normalized activity} = \frac{([\text{Ag}]/[\text{Ab}])_{\text{N or D}}}{([\text{Ag}]/[\text{Ab}])_{\text{N}}}$$

where [Ag] and [AL] are the surface concentrations of antigen and antibody, respectively, and the subscripts N and D represent native and denatured antibody cases, respectively. Values greater than 1 indicate that the apparent AgBC of the immobilized antibodies is higher than that of the native antibody. For agarose gels all values are close to 1 as expected, because the orientation of immobilized antibody was controlled by chemical coupling. Thus, it has the least dependence on the conformational changes. However, on silica surfaces all cases exhibit some degree of dependence on the adsorption properties of the antibodies. Positive orientational effects (cases b, c and d) and negative influences (case a) are observed.

In summary, we have demonstrated that exposure of antibodies to a low-pH buffer prior to immobilization results in an increase in AgBC of the immobilized antibody on silica surfaces. This can be a useful method to improve the column capacity in immunoaffinity chromatography. Another very important conclusion is that physical adsorption dominates the orientation of immobilized antibodies on silica surfaces. Although chemical bonds may be useful in providing stable linkages between the antibody and surface, they do not affect orientation of the antibody on the surface. This concept is applicable to all materials that have high non-specific adsorption sites for proteins, including antibodies and enzymes. Therefore, when such materials are chosen as the solid supports for affinity chromatography, one should not approach the problem totally on the basis of trial and error. Instead, one can either modify the antibody molecules in such a way that active sites have a low interaction energy to the surface, or modify the surface to preferentially interact with only certain parts of the antibody.

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